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Targeted sequencing of candidate gene regions for myelofibrosis in dogs

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Abstract

Background: Myelofibrosis often lacks an identifiable cause in dogs. In humans, most primary myelofibrosis cases develop secondary to driver mutations in *JAK2*, *CALR*, or *MPL*.

Objectives: To determine the prevalence of variants in *JAK2*, *CALR*, or *MPL* candidate regions in dogs with myelofibrosis and in healthy dogs.

Animals: Twenty-six dogs with myelofibrosis that underwent bone marrow biopsy between 2010 and 2018 and 25 control dogs matched for age, sex, and breed.

Methods: Cross-sectional study. Amplicon sequencing of *JAK2* exons 12 and 14, *CALR* exon 9, and *MPL* exon 10 was performed on formalin-fixed, decalcified, paraffin-embedded bone marrow (myelofibrosis) or peripheral blood (control) DNA. Somatic variants were categorized as likely-benign or possibly-pathogenic based on predicted impact on protein function. Within the myelofibrosis group, hematologic variables and survival were compared by variant status (none, likely-benign only, and ≥1 possibly-pathogenic). The effect of age on variant count was analyzed using linear regression.

Results: Eighteen of 26 (69%) myelofibrosis cases had somatic variants, including 9 classified as possibly-pathogenic. No somatic variants were detected in controls. Within the myelofibrosis group, hematologic variables and survival did not differ by variant status. The number of somatic variants per myelofibrosis case increased with age (estimate, 0.69; SE, 0.29; $P = .03$).

Conclusions and Clinical Importance: Somatic variants might initiate or perpetuate myelofibrosis in dogs. Our findings suggest the occurrence of clonal hematopoiesis in dogs, with increasing incidence with age, as observed in humans.

KEYWORDS
anemia, bone marrow, clonal hematopoiesis, myeloproliferative neoplasm, somatic mutation

Abbreviations: CHIP, clonal hematopoiesis of indeterminate potential; CI, confidence interval; MPN, myeloproliferative neoplasm; MPV, mean platelet volume; PCR, polymerase chain reaction; PIMA, precursor-targeted immune-mediated anemia; VAF, variant allele frequency; WBC, white blood cell.

1 | INTRODUCTION

In dogs, myelofibrosis is a poorly understood disease characterized by progressive replacement of bone marrow tissue with nonfunctional fibrous tissue. This process can occur as a sequela to bone marrow injury, with associated diseases including bone marrow toxic damage, immune-mediated disease, vascular damage, radiation damage, inflammation, pyruvate kinase deficiency, or neoplasia.[1] Classic clinical and histologic features consistent with primary myelofibrosis in humans (eg, splenomegaly, marked thrombocytosis, and megakaryocytic hyperplasia and dysplasia) are rarely present in dogs with myelofibrosis, and most cases are presumed to be secondary.[2] Immunosuppressive treatment results in long-term remission of anemia in 46% to 57% of cases.[3,4] Additionally, 48% to 49% of dogs with precursor-targeted immune-mediated anemia (PIMA) have myelofibrosis.[5,6] These data suggest that myelofibrosis often is associated with immune-mediated disease in dogs. However, it is not clear if a primary form of myelofibrosis also exists that might explain the inconsistent response to immunosuppressive treatment.

In humans, the diagnostic criteria for primary myelofibrosis have changed substantially over the last 2 decades with a shift from clinical and histologic features to prioritization of molecular features.[7,8] Primary myelofibrosis in humans is a myeloproliferative neoplasm (MPN) closely related to polycythemia vera and essential thrombocythemia.[9] Together, these 3 diseases comprise *BCR-ABL1*-negative MPN, a group of clonal disorders affecting the hematopoietic stem cells.[9] In humans, 90% to 95% cases of *BCR-ABL1*-negative MPN occur in association with somatic driver mutations in *JAK2*, *CALR*, or *MPL*.[10-15] These mutations hyperactivate the JAK2/STAT5 transcription factor pathway, which regulates hematopoiesis, and constitutive activity leads to dysregulated cell proliferation and a neoplastic phenotype.[10-12,15,16] Acquisition of a *JAK2*, *CALR*, or *MPL* driver mutation is specific for MPN; they are rare in individuals with other cancer types.[17-19] Accordingly, detection of a mutation in *JAK2*, *CALR*, or *MPL* is a major diagnostic criterion in the 2016 revision to the World Health Organization guidelines for MPN.[8] With new understanding of the molecular etiology of primary myelofibrosis in humans, the frequency and potential molecular etiology of this disease in dogs must be reassessed using revised criteria and techniques. Knowledge of the occurrence and frequency of primary myelofibrosis might inform diagnostic recommendations and management.

In 2011, a *JAK2* V617F mutation was identified in 1 of 5 dogs with suspected polycythemia vera.[20] The *JAK2* V617F mutation is the most common driver of polycythemia vera and myelofibrosis in humans.[7] Its discovery in a dog suggests that MPN might share an underlying pathogenesis across species. Therefore, our aim was to determine the prevalence of somatic *JAK2*, *CALR*, and *MPL* mutations in dogs with collagen myelofibrosis, as well as in healthy control dogs. Our hypothesis was that driver mutations would exist in dogs with collagen myelofibrosis but not in healthy controls, supporting a neoplastic etiology.

2 | METHODS

2.1 | Sample size calculation

A sample size calculation was performed to determine the number of dogs with myelofibrosis needed to achieve ≥80% probability of detecting somatic variants in MPN candidate driver gene regions if the true population prevalence is ≥10%. Using the formula $1 - (0.9)^n$ for probability of an event, we determined that we needed to sequence at least 16 dogs with myelofibrosis.

2.2 | Sample selection

Cases were identified by means of an electronic medical records query for dogs that had bone marrow biopsy performed at the University of Minnesota Veterinary Medical Center between 2010 and 2018. Bone marrow aspirate cytology and core biopsy histopathology samples were reviewed concurrently by the board-certified veterinary pathologist on service. Samples for histopathology were evaluated using hematoxylin and eosin staining, and dogs with any degree of collagen fibrosis were included. Samples were not routinely stained with Masson's trichrome and were not stained for reticulin fibrosis. Pathologist reports of the bone marrow biopsies were reviewed by a single investigator (Eva Furrow), masked to outcome and somatic variant status, to extract information on the fibrosis grade. Samples with an unconfirmed diagnosis of myelofibrosis, as indicated by a pathologist description such as "possible early myelofibrosis," "possible reticulin fibrosis," or "very focal mild fibrosis," were excluded. Samples with myelofibrosis described as "mild," "a small amount," or "mild-to-moderate" were classified as having mild fibrosis, and samples with myelofibrosis described as "moderate," "moderate-to-marked," "marked," "extensive," or "significant" were classified as having moderate-to-marked fibrosis. Medical records for the included cases were reviewed and the following data recorded: breed, sex, date of birth, age at time of bone marrow biopsy, bone marrow aspirate and core biopsy findings, main differential diagnoses, current medications at the time of bone marrow biopsy, treatments instituted after performing bone marrow biopsy, case outcome, and survival time. Data also were extracted from the CBC performed within 24 hours of the bone marrow biopsy. The CBC and bone marrow core biopsy reports were used to determine if dogs met criteria for suspect PIMA, including nonregenerative anemia and ineffective hematopoiesis (erythroid hyperplasia or increase in early-stage erythroid precursors with evidence of maturation arrest) without known neoplasia, drug reaction, or other major concurrent disease.[5] Nonregenerative anemia was defined as a hematocrit ≤30% and reticulocyte count <81 500/μL (upper limit of the laboratory reference interval) for ≥5 days.

Because bone marrow biopsies were not available for healthy dogs, a comparison population was obtained from a biobank of canine peripheral blood DNA samples at the University of Minnesota Canine Genetics Laboratory. In humans, driver mutations of myeloproliferative neoplasms are detected in bone marrow and peripheral blood

DNA at nearly equivalent variant allele frequency (VAF).[21-23] Samples were selected from dogs without known hematologic disease (determined by a health survey completed by the submitting veterinarian that asked if the dog had a "blood/lymph disorder") that were the closest available match to each case by age and breed. Hematologic data was reviewed, if available within 1 month of obtaining the blood sample (Supplementary Table 1). One exception to the requirement for absence of hematologic disease was a dog with multicentric large cell lymphoma without cytopenias, which was selected to match a myelofibrosis case with multicentric large cell lymphoma without bone marrow invasion.

Institutional Animal Care and Use Committee approval was not required for use of the bone marrow samples, because they were collected as part of routine veterinary care. The banked peripheral blood DNA was collected under multiple protocols previously approved by the University of Minnesota Institutional Animal Care and Use Committee (1809-36373A, 1807-36213A, 1509-33019A, 1207A-17243) and through submissions to the University of Minnesota Canine Genetics Laboratory for diagnostic testing.

2.3 | Amplicon sequencing and variant detection

Genomic DNA was extracted from formalin-fixed, decalcified (10% EDTA for 2-3 hours), paraffin-embedded bone marrow biopsy samples using *Hemo*-De (*d*-limonene; Electron Microscopy Sciences, Hatfield, Pennsylvania) and the Gentra Puregene blood kit (Qiagen, Germantown, Maryland). Peripheral blood DNA previously had been extracted and stored at −20°C until use. Primers were designed to amplify 185 to 250 base pair products for 4 target regions (the location of canonical driver variants of primary myelofibrosis in humans: *JAK2* exons 12 and 14, *CALR* exon 9, and *MPL* exon 10)[9-15] with partial Illumina adaptors (Supplementary Table 2). Polymerase chain reaction (PCR) was performed utilizing HotStarTaq DNA Polymerase (Qiagen, Germantown, Maryland) with "slowdown PCR" cycling conditions.[24] Products were purified using Illustra ExoProStar (Cytiva, Marlborough, Massachusetts).

The DNA library preparation and amplicon sequencing reactions were performed at GENEWIZ, Inc. (South Plainfield, New Jersey). A NEBNext Ultra DNA Library Prep kit (Illumina, San Diego, California) was used for DNA library preparation and clustering, according to the manufacturer's recommendations. Amplicons were indexed and enriched by limited cycle PCR. The DNA libraries were validated on the Agilent TapeStation (Agilent Technologies, Palo Alto, California), quantified using Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, California), and multiplexed in equal molar mass. The pooled DNA libraries were loaded and sequenced on an Illumina MiSeq, using a 2 × 250 paired-end configuration.

Raw Illumina reads were checked for quality using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimmed of adapters and poor quality nucleotides using Trimmomatic version 0.36.[25] Reads then were mapped to canine reference genomes using Bowtie 2, Galaxy version 2.4.2.[26] Both canFam3.1 and UU_Cfam_GSD_1.0 reference genomes were used, because some of the downstream annotation tools were only available for canFam3.1, but *MPL* exon 10 resides in a gap in canFam3.1 that was filled in UU_Cfam_GSD_1.0. Reads were filtered for those that mapped in a proper pair using the SAMTools toolkit. Mate-pair information was verified and fixed using Picard tools (https://broadinstitute.github.io/picard), and local realignment was performed around indels using SAMTools toolkit.[27]

2.4 | Variant calling and prioritization

Variants were detected using FreeBayes, Galaxy version 1.3.1.[28] The minimum VAF was set to 2% based on the lower limit observed in myelofibrosis in humans and to minimize the probability of false positive calls from formalin fixation artifacts.[29,30] Variants were filtered for quality by excluding those with base and mapping quality scores <30 and for strand bias by excluding those with a ratio of forward to reverse strand frequencies outside of the 99% probability estimate for departure from 0.5. Only variants with a minimum of 50 supporting reads were included. Variants were categorized as somatic or likely germline, the latter of which was defined as a VAF of 49% to 51% and a ≥1% allele frequency in the variant catalogue of the Dog Biomedical Variant Database Consortium.[31] At the time of the study, this catalogue contained whole genome sequencing variant calls from 813 dogs of 134 breeds. Variants were annotated using SnpEff.[32] Those with putative moderate to high impact on the protein sequence (nonsense, missense, frameshift, inframe deletion or insertion, and splice site variants) were categorized as possibly-pathogenic. Missense variants were further analyzed using the pathogenicity prediction programs SIFT (run via the Variant Effect Predictor for variants mapped to canFam3.1) and Provean.[33-35] Missense variants predicted to be benign by both programs were excluded from the list of possibly-pathogenic variants. Variants were checked for presence in the Catalogue Of Somatic Mutations In Cancer (COSMIC),[36] and information on the cancer type was recorded for any previously reported variants. Variants were classified as canonical drivers if previously reported in association with myelofibrosis in humans, such as *JAK2* V617F, *CALR* indels, and *MPL* W513 substitutions (homologous to human W515).[9]

2.5 | Statistical analysis

Descriptive data are summarized as median and range. Counts and proportions of total somatic variants and possibly-pathogenic variants were determined for the myelofibrosis cases and peripheral blood controls. Within the myelofibrosis group, Kruskal-Wallis rank sum tests were used to compare hematocrit, platelet concentration, and white blood cell (WBC) count at the time of bone marrow biopsy and the highest hematocrit recorded during follow-up assessments among 3 ordered subsets with: (1) no somatic variants detected, (2) likely-benign variants only, and (3) ≥1possibly-pathogenic variants. Kaplan-Meier survival curves were generated with right censoring to estimate

median survival times and 95% pairwise confidence intervals (CIs). Survival was compared among the 3 subsets of myelofibrosis cases using the log-rank test. A Cox regression was performed to quantify the effect of variant subset, hematocrit, platelet count, WBC count, myelofibrosis grade (mild vs moderate-to-severe) and age on time to death. A Chi-square test for trends in proportions was used to compare the proportion of dogs with moderate-to-severe fibrosis as well as the proportion meeting criteria for suspect PIMA among the ordered variant subsets (none, likely-benign only, and possibly-pathogenic). Linear regression was performed to determine if the total number of somatic variants changed with age within the myelofibrosis group. The total number of somatic variants also was compared between sexes (males vs females) using a Fisher's exact test. Statistical analyses were performed using R software for statistical computing (R, version 4.1.1. www.r-project.org). *P* values are presented without inference of significance, consistent with the American Statistical Association's Statement on *P*-values.[37]

3 | RESULTS

3.1 | Animals

One-hundred and eighty-seven dogs had bone marrow biopsies performed during the study time frame. Of those, 26 met the inclusion criteria and were selected as myelofibrosis cases for the study (Figure 1). Signalment data for the case group is presented in Table 1. Twenty-six dogs initially were selected as the peripheral blood DNA comparison group; subsequently, 1 control dog (a 9-year-old female Boston Terrier) was excluded from analysis because of neutropenia, leaving a total of 25 control dogs. Hematologic data was available from 11 of the control dogs (summarized in Supplementary Table 1). Sex, age, and breed for the case and comparison groups were matched as closely as possible (Table 1).

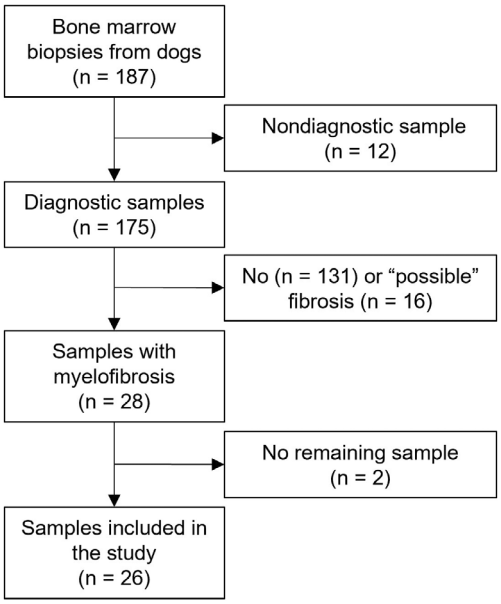

FIGURE 1 Flowchart indicating selection of bone marrow biopsy samples from dogs for *JAK2*, *CALR*, and *MPL* target region sequencing.

TABLE 1 Signalment data for 26 dogs with collagen myelofibrosis (cases) and 25 dogs without hematologic disease (controls)

Variable	Myelofibrosis cases	Peripheral blood controls
Sex	77% (20) female 23% (6) male	64% (16) female 36% (9) male
Age (y)	7.7 y (range, 3.4-12)	8.0 y (range, 2.9-10.7)
Breeds	19% (5) Labrador Retriever 12% (3) Dachshund 8% (2) Shih Tzu 8% (2) Beagle 8% (2) American Staffordshire Terrier 8% (2) Boston Terrier 38% (10) Other breeds (n = 1 for each)	20% (5) Labrador Retriever 12% (3) Dachshund 8% (2) Shih Tzu 8% (2) Beagle 8% (2) Staffordshire Bull Terrier 44% (11) Other breeds (n = 1 for each)

3.2 | Hematologic characteristics, bone marrow findings, and clinical outcome of myelofibrosis cases

Results of CBCs performed within 24 hours of the bone marrow sampling are summarized in Table 2. All 26 dogs were anemic, with 22/26 (85%) dogs having nonregenerative anemia. Other common abnormalities were increased mean platelet volume (MPV, 20, including 11 with platelet counts within reference intervals, 6 with thrombocytosis, and 3 with thrombocytopenia); left shift of neutrophils (10); monocytosis (10); thrombocytosis (8); and neutrophilia (8). The most common abnormalities on peripheral blood smear slide review were anisocytosis (21), polychromasia (18), reactive lymphocytes (11), and macrocytosis (10).

In the bone marrow histopathological descriptions, the degree of myelofibrosis was classified as mild in 13 cases and moderate-to-marked in 13 cases. Twenty-one of 26 (81%) dogs had ineffective erythropoiesis. Other histopathological findings included edema (13), erythroid dysplasia (8, mild for all and concurrent with ineffective erythropoiesis for all but 1), necrosis (5), myeloid hyperplasia (4), myeloid hypoplasia (3), megakaryocytic hyperplasia (2), erythroid hypoplasia (2), megakaryocytic dysplasia (2, mild for both), myeloid dysplasia (1, mild), lymphoplasmacytic infiltration (1), suppurative inflammation (1, mature neutrophils without earlier precursors), cytophagic macrophages (1, containing brown, granular material consistent with hemosiderin or apoptotic cell fragments), and possible hematopoietic neoplasia (1). Nineteen (73%) of the dogs met the criteria for suspected PIMA, but myelofibrosis was listed as the main differential diagnosis for 5 in the pathologist's report. The 19 dogs with suspected PIMA included 2 with a history of nonregenerative anemia (>4 weeks' duration), on immunosuppressive treatment, and with mild reticulocytosis at the time of the bone marrow biopsy. Two additional dogs had PIMA as the main differential diagnosis in the report but did not meet PIMA criteria because of reticulocytosis (1) or inability to categorize the anemia as nonregenerative because of unknown duration (1).

TABLE 2 Hematologic abnormalities in 26 dogs with collagen myelofibrosis

Variable	Laboratory reference interval (RI)	Case median (range)	Proportion above URL (H) and below LRL (L)
Hematocrit	37.5%-60.3%	17.2% (7.8-36.1)	H: 0/26 (0%) L: 26/26 (100%)
MCV	63.0-75.3 fL	72 (59.9-85.7)	H: 6/26 (23%) L: 1/26 (4%)
MCHC	33.6-37.4 g/dL	32.8 (29.2-36.2)	H: 0/26 (0%) L: 18/26 (69%)
Reticulocyte count	6-82 k/μL	29.5 (3-120)	H: 4/26 (15%) L: 3/26 (12%)
Platelet count	129-395 k/μL	268 (14-1052)	H: 8/23 (35%)[a] L: 3/23 (13%)[a]
MPV	8.1-13.7 fL	21.2 (13.4-29.5)	H: 19/22 (86%)[a] L: 0/22 (0%)[a]
WBC count	3.88-14.57 k/μL	11.3 (1.3-34)	H: 7/26 (27%) L: 3/26 (12%)
Segmented neutrophils	2.1-11.2 k/μL	8.2 (0.8-27.5)	H: 8/26 (31%) L: 3/26 (12%)
Band neutrophils	0-0.13 k/μL	0.1 (0-2.4)	H: 10/26 (38%) L: NA
Lymphocytes	0.78-3.36 k/μL	1.2 (0.2-3.7)	H: 1/26 (4%) L: 8/26 (31%)
Monocytes	0-1.2 k/μL	0.6 (0-3.3)	H: 10/26 (38%) L: NA
Eosinophils	0-1.2 k/μL	0.0 (0-0.8)	H: 0/26 (0%) L: NA
Basophils	0-0.13 k/μL	0 (0)	H: 0/26 (0%) L: NA
nRBCs (#/100 WBC)	0	0.8 (0-7)	H: 7/26 (27%) L: NA

Abbreviations: MCV, mean corpuscular volume; MPV, mean platelet volume; nRBC, nucleated red blood cells; WBC, white blood cell.
[a]The total number of dogs is 23 instead of 26 because of platelet clumps that precluded an estimate of platelet count or MPV for 3 dogs.

Myelofibrosis was reported as the main differential diagnosis for 4 cases that did not meet PIMA criteria because of erythroid hypoplasia (2) or normal cellularity (2) without an increase in erythroid progenitors. One case had a main differential diagnosis of hematopoietic neoplasia because of the presence of medium- to large-sized mononuclear cells that could have represented neoplastic lymphocytes or histiocytes.

Twelve dogs were receiving immunosuppressive corticosteroid treatment (prednisone or prednisolone) at the time of the bone marrow biopsy, with dosages ranging from 1 to 2 mg/kg per day. Six dogs received a second immunosuppressive drug, including azathioprine (3) and mycophenolate (3). After bone marrow biopsy, 25 dogs were treated with corticosteroids (prednisone or prednisolone). Of those, 12 dogs received the corticosteroids as the sole immunosuppressive treatment, and 12 received corticosteroids in combination with a second immunosuppressive agent: cyclosporine (4), mycophenolate (5), or azathioprine (3). The other dog treated with corticosteroids also concurrently received multiagent chemotherapy because of a new diagnosis of large cell lymphoma (this dog had suspect hematopoietic neoplasia in the bone marrow). One dog was euthanized without further treatment.

Median survival for dogs with myelofibrosis was 158 days (95% CI, 98 to undefined). Of the 25 dogs treated with immunosuppressive drugs, 7 (28%, including the dog that received multiagent chemotherapy) responded with normalization of hematocrit at a median of 238 days (range, 14-856 days), 17 (68%) had persistent anemia with a median hematocrit of 19% (range, 9%-30%) at a median follow-up of 23 days (range, 3-1286 days), and 1 (4%) did not have a follow-up hematocrit available.

3.3 | Sequencing results

The average depth of mapped reads across target regions was 36 614 ± 16 880 reads. No germline variants were detected. Sixty-three somatic variants met the filtering criteria, including 21 in *JAK2*, 23 in *CALR*, and 19 in *MPL* target regions. There were 22 synonymous, 22 missense, 11 intronic, 6 untranslated region, and 2 nonsense variants detected (see Supplementary Table 3). Somatic variants were detected in 18/26 (69%) myelofibrosis cases. Four of the variants were present in 2 cases each; the other 59 were identified only in a single case. No somatic variants were detected in the peripheral blood

TABLE 3 Somatic variants with predicted pathogenicity discovered in 6 of 26 dogs with collagen myelofibrosis that underwent bone marrow sample sequencing for candidate regions of *JAK2*, *CALR*, and *MPL*

Gene	Variant	VAF	SIFT call (score)[a]	PROVEAN call (score)[a]
JAK2	P524L	2.5%	Deleterious (0)	Deleterious (−3.3)
	F595L	3.6%	Tolerated (0.49)	Deleterious (−3.2)
	Q603*	9.1%	NA (nonsense)	NA (nonsense)
	L604P	2.3%	Deleterious (0)	Deleterious (−4.3)
	H606Y	3.6%	Tolerated (0.39)	Deleterious (−3.8)
	G619E	2.4%	Deleterious (0.04)	Deleterious (−4.1)
CALR	Q361*	3.4%	NA (nonsense)	NA (nonsense)
	D362G	4.1%	Deleterious (0.04)	Deleterious (−5.7)
MPL	P516L	2.6%	Tolerated (1)	Deleterious (−2.8)

Abbreviations: NA, not applicable; VAF, variant allele frequency.
[a]SIFT scores ≤0.05 and PROVEAN scores ≤−2.5 are predicted to be deleterious. Neither program provides calls for nonsense mutations; these are loss-of-function mutations and were thus automatically predicted to have pathogenicity.

control samples at the specified VAF of ≥2%. For myelofibrosis cases with somatic variants detected, the median number of variants per case was 2 (range, 1-11), and the median VAFs were 3.6% (range, 2%-40.7%), 2.9% (range, 2.1%-53.5%), and 3.2% (range, 2.4%-19.2%) for variants detected in *JAK2*, *CALR*, and *MPL*, respectively.

Nine of the somatic variants, present in 6/26 (23%) myelofibrosis cases, were classified as possibly-pathogenic based on predicted effect on protein function. All 6 cases had a possibly-pathogenic *JAK2* variant, with a coexisting *CALR* variant in 2 dogs and an *MPL* variant in 1 dog. The degree of myelofibrosis was mild in 5 cases and moderate-to-marked in 1 case. Other histopathologic findings in the bone marrow of these 6 dogs were erythroid hyperplasia (5), erythroid dysplasia (4), megakaryocytic hyperplasia (2), megakaryocytic hypoplasia (1), and myeloid hypoplasia (1). The dogs had main differential diagnoses of PIMA (4), idiopathic myelofibrosis (1), and possible hematopoietic neoplasia (1). All 6 dogs were treated with immunosuppressive drugs, and 2/6 (33%) had normalization of their hematocrit during follow-up evaluations. Variant details, including VAFs, are presented in Table 3. All of these possibly-pathogenic variants were transitions resulting in missense (7) or nonsense (2) mutations.

Three of the somatic variants were reported in cancer samples from human patients in the COSMIC database. These included a possibly-pathogenic *JAK2* L604P variant reported in prostatic carcinoma, a possibly-pathogenic *MPL* P516S (homologous to human P518S) variant reported in cervical squamous cell carcinoma, and a likely-benign JAK2 S518 = variant in polycythemia vera. The canonical driver variants *JAK2* V617F, *CALR* indels, and *MPL* W513L/K were not identified.

3.4 | Comparison of myelofibrosis cases with and without somatic variants

Hematocrit, WBC count, and platelet count at the time of bone marrow biopsy did not differ among myelofibrosis cases with no variants identified, those with likely-benign variants only, and those with ≥1 possibly-pathogenic variants (Figure 2, Supplementary Table 4). The 2 dogs with megakaryocytic hyperplasia had possibly-pathogenic variants. One dog with megakaryocytic dysplasia had a likely-benign variant, and the other (that also had erythroid and granulocytic dysplasia) had no variants detected. The proportion of dogs with moderate-to-severe fibrosis decreased among variant groups with the highest proportion in dogs with no variants (6/8), followed by those with likely-benign only (6/12), and then possibly-pathogenic (1/6; $P = .03$) variants. No difference was detected in the proportion of dogs meeting PIMA criteria among variant groups (6/8 dogs with no variants, 8/12 with likely-benign only, and 5/6 with possibly-pathogenic variants; $P = .78$). The highest hematocrit recorded during follow-up assessments did not differ among groups (Supplementary Table 4). Survival also did not differ among groups (Figure 3). Median survival times were 514 days (95% CI, 98 to undefined) for dogs with no somatic variants, 152 days (95% CI, 29 to undefined) for dogs with likely-benign variants, and 644 days (95% CI, 129 to undefined) for dogs with possibly-pathogenic variants. Results of the Cox regression model are shown in Table 4; none of the tested predictors affected time to death.

3.5 | Predictors of somatic variant burden in myelofibrosis cases

Within the myelofibrosis group, the total number of somatic variants detected per dog increased with age (Figure 4). The number of somatic variants was not different between males and females (mean, 2.7 ± 3.3 vs 2.6 ± 3.1, respectively; $P = .94$).

4 | DISCUSSION

In our study of bone marrow samples from 26 dogs with myelofibrosis, we identified 9 possibly-pathogenic and 54 likely-benign somatic variants in the candidate regions of *JAK2*, *CALR*, and *MPL*. Some of

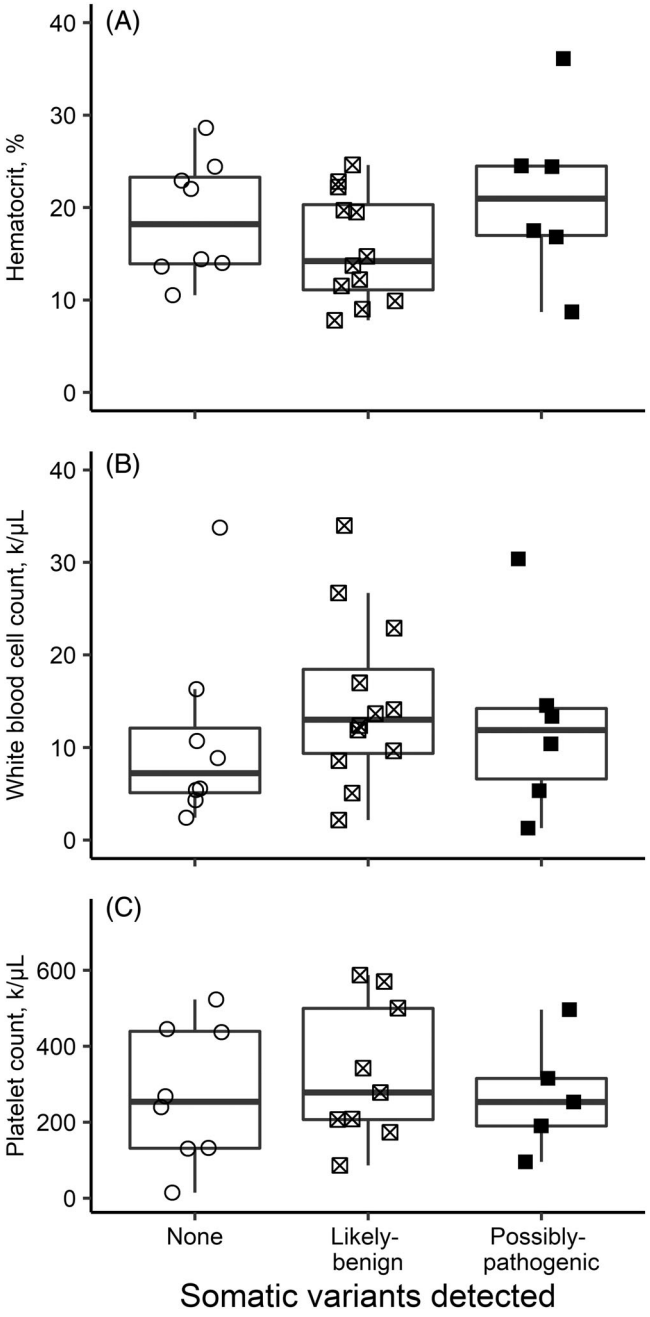

FIGURE 2 Box and whisker plots of (A) hematocrit, (B) white blood cell count, and (C) platelet count for 26 dogs with collagen myelofibrosis screened for somatic variants in target regions of *JAK2*, *CALR*, and *MPL* in bone marrow samples. Dogs with no somatic variants detected are represented with open dots, dogs with likely-benign variants only are represented with crossed squares, and dogs with possibly-pathogenic variants are represented with filled squares. The boxes represent the interquartile range, and the whiskers represent 1.5 times the interquartile range. Hematocrit ($P = .32$), white blood cell count ($P = .51$), and platelet count ($P = .54$) did not differ between dogs with no variants, likely-benign variants only, and ≥1 possibly-pathogenic variants.

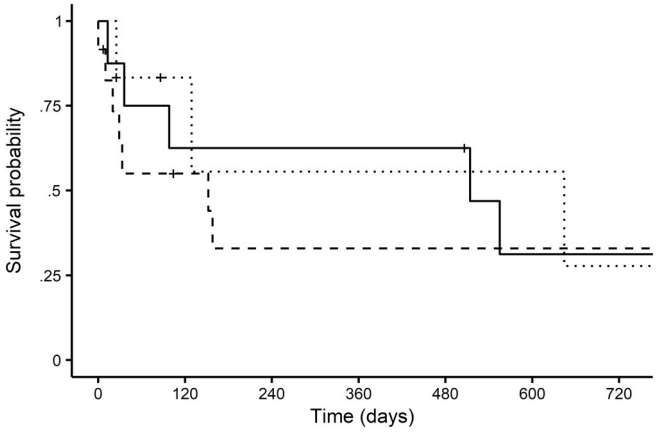

FIGURE 3 Kaplan-Meier survival curves for 26 dogs with collagen myelofibrosis, comparing those without any somatic variants detected (solid line, n = 8), likely-benign variants only (dashed line, n = 12) and ≥1 possibly-pathogenic variants (dotted line, n = 6) detected in target regions of *JAK2*, *CALR*, and *MPL* in bone marrow samples. Survival curves did not differ between groups ($P = .78$). Crosses represent censored observations.

the somatic variants detected might act as initiators of the myelofibrotic process, but most variants were present at relatively low VAF (<10%) and not associated with decreased survival. However, we did not detect the canonical driver variants of primary myelofibrosis in humans, and therefore our study does not provide direct evidence of primary myelofibrosis in dogs. Of note, the total number of somatic variants per dog increased with age. In humans, accumulation of somatic variants within hematopoietic cells is a common phenomenon observed with aging termed "clonal hematopoiesis."[38] No somatic variants were detected in the peripheral blood samples from 25 dogs without hematologic disease, despite age similar to that of the myelofibrosis cases. This observation suggests that the higher total somatic variant burden in myelofibrosis cases might be a sequela of hematologic pathology within the bone marrow in addition to aging.

In humans, primary myelofibrosis is thought to be initiated by driver mutations that lead to constitutive signaling of the JAK2/STAT5 intracellular signaling pathway. Of the possibly-pathogenic variants identified in our study, 4 were *JAK2* missense variants at residues where mutation is hypothesized to destabilize the interaction between the JAK2 protein's JH1 and JH2 domains, required for hyperactive signaling of the JAK2 pathway.[39,40] Two of these mutations, L604P and H606Y, might alter the conformation of a critical loop, changing the position of V617 relative to a C-helix.[41] In support of this possibility, another substitution at 1 of these residues (H606Q) was identified in a human patient with suspected MPN.[42] Thus, ≥1 of the *JAK2* variants identified could act in a way similar to the *JAK2* V617F mutation, resulting in hyperactivation of the protein's signaling apparatus. A limitation of our study is that the functional effects of these newly identified mutations were not determined. Additional studies investigating the impact of these mutations on JAK2/STAT5 signaling at the cellular level would be needed to establish whether or not they have a similar effect to the driver mutations reported in humans with MPN, and thus whether or not any of the study cases truly represents diseases analogous to BCR-ABL1-negative MPN.

TABLE 4 Cox regression model for the effect of bone marrow somatic variants in *JAK2*, *CALR*, and *MPL* candidate regions, hematologic variables, age, and degree of fibrosis (mild vs moderate-to-marked) on time to death in 23 dogs with collagen myelofibrosis

Predictors	Hazards ratio	95% CI	*P* value
Likely-benign variants	2.11	0.47-8.55	.3
Possibly-pathogenic variants	0.76	0.1-5.57	.79
Age	0.91	0.61-1.38	.67
Hematocrit	1.04	0.93-1.16	.45
WBC count	1.05	0.97-1.13	.21
Platelet count	1	0.99-1	.15
Moderate-to-marked fibrosis	0.58	0.15-2.34	.45

Abbreviations: CI, confidence interval; WBC, white blood cell.

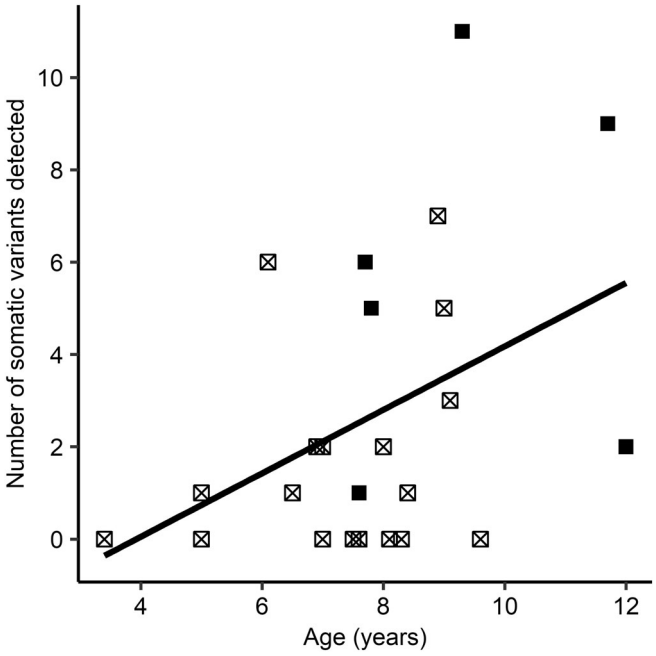

FIGURE 4 Age correlates with the number of somatic variants detected in target regions of *JAK2*, *CALR*, and *MPL* in bone marrow samples from 26 dogs with collagen myelofibrosis (estimate of the coefficient = 0.69, SE = 0.29, $P = .03$). The crossed squares represent likely-benign variants, and the filled squares represent possibly-pathogenic variants.

In our study, the VAF of pathogenic variants ranged from 2% to 9%. This percentage is lower than what is typically observed in humans with primary myelofibrosis, in whom the average VAF is 31%-67% for *JAK2*, 38%-50% for *CALR*, and 25% for *MPL* driver mutations.[43-46] The variant with the highest VAF in our study, JAK2 Q603*, results in a nonsense mutation, most likely resulting in a loss of function for the protein product via nonsense-mediated decay.[47] This outcome would not be consistent with a driving mutation for MPN, which results from gain of function mutations in *JAK2*. However, we cannot rule out that this protein product could be escaping nonsense-mediated decay and contributing to the disease process.[48] Regarding the other possibly-pathogenic variants, despite the relatively low VAFs, some could be consistent with a diagnosis of MPN. There is no established VAF cut off for the diagnosis of a MPN, and burdens as low as 0.8% have been proposed.[49] Alternatively, despite strict quality control measures, some low frequency variants might be artifacts. Although the sensitivity of somatic variant detection is excellent in formalin-fixed tissue samples,[21,23,50,51] formalin fixation introduces mutations, most of which are a result of cytosine deamination.[30,51,52] The false discovery rate in formalin-fixed tissue is highest for variants with VAF <2% to 5%.[30,52] Verification of findings using fresh bone marrow or peripheral blood samples would be ideal but was not possible, given the use of archived samples.

The 6 dogs with possibly-pathogenic variants had a variety of bone marrow histopathological diagnoses, including PIMA (4), idiopathic myelofibrosis (1), and potential hematopoietic neoplasia (1). They did not clearly represent a unique subpopulation within our test group of dogs with myelofibrosis. The group with possibly-pathogenic variants included the only 2 dogs with megakaryocytic hyperplasia, but neither of the 2 with megakaryocytic dysplasia reported. Hematological variables, such as hematocrit, WBC count, platelet count, and the highest hematocrit achieved during follow-up assessments, did not differ from dogs with likely-benign or no variants. Unexpectedly, the proportion of dogs with moderate-to-severe fibrosis was higher in those without variants than in those with likely-benign variants and lowest in those with possibly-pathogenic variants. Collagen fibrosis grade varies in dogs with myelofibrosis, whether or not they have PIMA,[2-6] and severity does not predict outcome.[5,6] In humans with primary myelofibrosis, a higher grade is associated with shorter survival.[53,54] In our study, survival was not predicted by fibrosis severity nor by variant status. However, 95% CIs were wide (with undefined upper limits) because of the small number of dogs in each subgroup. The observation of a similar outcome in dogs without possibly-pathogenic variants does not necessarily preclude an underlying neoplastic etiology. In fact, corticosteroid treatment, which remains the primary treatment in most veterinary patients with immune-mediated disease, also is used to treat the anemia associated with myelofibrosis in people.[55] However, the variants detected are not conclusive evidence for primary myelofibrosis in dogs.

Another explanation for the detection of variants in myelofibrosis cases is that some are the result of an abnormal bone marrow microenvironment rather than drivers of disease. It is hypothesized that chronic bone marrow inflammation attenuates the hematopoietic stem cell progenitor pool over time, because inflammatory cytokine cascades damage and prematurely age progenitor cells, while those with a selective advantage expand to fill the void.[38,56,57] This phenomenon also could explain the lack of mutations in our healthy control dogs, which had no known hematologic abnormalities. Unfortunately, CBC data was only available from 11 control dogs, and bone marrow samples were not available from any of them to verify the absence of bone marrow pathology. The use of different samples for genetic analysis from controls vs cases is also a limitation of the study. In humans, the presence and VAF of MPN drivers and other

somatic variants are nearly equivalent for peripheral blood and bone marrow samples.[21-23,58] Therefore, we do not expect the discrepancy in sample origin between cases and controls to be caused by false negative calls in the controls. More likely, the variants discovered in the myelofibrosis group are driver mutations, a sequela of the underlying disease process, or possibly artifacts of formalin fixation. However, it would be worthwhile in future studies to confirm that peripheral blood and bone marrow samples show comparable results in dogs, as they do in humans.

Although we did not determine the functional impact of the somatic variants discovered in our study, their presence suggests that clonal hematopoiesis occurs in dogs. Clonal hematopoiesis of indeterminate potential (CHIP) refers to an expansion of mutant clonal hematopoietic stem cells in the absence of overt hematologic disease.[38] It is defined as a somatic variant in hematopoietic cells with VAF > 2% and includes variants in candidate driver genes associated with the development or promotion of hematopoietic neoplasia.[38] Proposed mechanisms for development of CHIP include aging, chronic inflammation, exogenous genotoxic stress (eg, chemotherapy exposure), and bone marrow immune-mediated disease.[38] Over time, these conditions might contribute to the selection of mutant clones with a survival advantage.[38] Indeed, CHIP is highly age-dependent, with a striking gradient when subjects are stratified by age. Its prevalence increases from <1% for humans <35 to 40 years of age to >50% for those older than 85 years.[59] In our study, the number of somatic variants detected in the candidate driver genes increased with age in dogs with myelofibrosis. However, the age-matched control group did not have any variants at or above the 2% VAF threshold. Because all variants detected occurred in dogs with hematologic disease, we cannot use the term CHIP. Our findings however suggest that, as in people, clonal hematopoiesis might occur as a multifactorial process, with both aging and chronic inflammation in the bone marrow contributing to its development in dogs. Additionally, we selected our target regions based on the causes of primary myelofibrosis in people. We did not screen the genes most commonly associated with CHIP in humans (*DNMT3A*, *TET2*, and *ASXL1*),[58-60] because doing so was not an aim of our study. Our older control dogs and myelofibrosis cases may likely have variants consistent with CHIP in these genes or in others that were not assessed.

In humans, CHIP confers an up to 10-fold increased risk of developing hematologic cancer (eg, leukemia, myelodysplastic syndrome, and MPN), as well as increased risk of coronary heart disease, ischemic stroke, venous thromboembolism and all-cause mortality.[60,61] The finding of apparent clonal hematopoiesis in dogs with myelofibrosis raises the question of whether affected dogs are predisposed to developing CHIP-associated complications in the future. Most dogs in our study met criteria for PIMA, an inflammatory bone marrow condition. Dogs with PIMA are at risk of thromboembolic events.[5] Clonal hematopoiesis that activates the JAK2/STAT5 pathway increases thrombus formation by platelet aggregation, among other mechanisms.[57] This finding introduces the possibility that the accumulation of mutant clones contributes to the thromboembolic risk observed in PIMA.

Limitations of our study were that it spanned a relatively long timeframe, the samples were not stained with Masson's trichrome for definitive diagnosis, and the samples were not all evaluated by a single pathologist. Although the long timeframe is not expected to alter sequencing results (use of archived bone marrow samples has similar sensitivity and specificity to use of fresh samples),[21] it might have affected the pathologist's interpretation of the bone marrow findings. To account for this possibility, we applied standardized criteria for the suspicion of PIMA.[5] No differences were found in the proportion of dogs meeting the PIMA criteria among variant categories. Because none of the variants were canonical driver mutations, it is not possible to conclude whether their presence indicates that some suspect PIMA cases were in fact primary myelofibrosis cases.

In summary, our study identified a high prevalence of somatic variants in candidate regions of *JAK2*, *CALR*, and *MPL* in dogs with myelofibrosis with a positive correlation between age and the number of variants detected per dog. This finding suggests that clonal hematopoiesis occurs in dogs and has an age-dependent manifestation, as in humans. Some of the somatic variants identified are possible drivers of MPN, but this possibility cannot be resolved without functional studies, because none of the identified variants in dogs are canonical driver mutations in humans. Our study likely overestimated the true prevalence of somatic variants in these target regions in dogs with myelofibrosis, because some low frequency mutations might be artifacts of formalin fixation.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Collection of peripheral blood DNA was approved by the University of Minnesota IACUC under multiple protocols (1809-36373A, 1807-36213A, 1509-33019A, 1207A-17243). Authors declare that IACUC approval was not required for use of the bone marrow samples, as they were collected as part of routine veterinary care.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

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